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RESEARCH ARTICLE

Open Access



# *Caenorhabditis monodelphis* sp. n.: defining the stem morphology and genomics of the genus *Caenorhabditis*

Dieter Slos<sup>1\*</sup> , Walter Sudhaus<sup>2</sup>, Lewis Stevens<sup>3\*</sup>, Wim Bert<sup>1</sup> and Mark Blaxter<sup>3</sup>

## Abstract

**Background:** The genus *Caenorhabditis* has been central to our understanding of metazoan biology. The best-known species, *Caenorhabditis elegans*, is but one member of a genus with around 50 known species, and knowledge of these species will place the singular example of *C. elegans* in a rich phylogenetic context. How did the model come to be as it is today, and what are the dynamics of change in the genus?

**Results:** As part of this effort to “put *C. elegans* in its place”, we here describe the morphology and genome of *Caenorhabditis monodelphis* sp. n., previously known as *Caenorhabditis* sp. 1. Like many other *Caenorhabditis*, *C. monodelphis* sp. n. has a phoretic association with a transport host, in this case with the fungivorous beetle *Cis castaneus*. Using genomic data, we place *C. monodelphis* sp. n. as sister to all other *Caenorhabditis* for which genome data are available. Using this genome phylogeny, we reconstruct the stem-species morphological pattern of *Caenorhabditis*.

**Conclusions:** With the morphological and genomic description of *C. monodelphis* sp. n., another key species for evolutionary and developmental studies within *Caenorhabditis* becomes available. The most important characters are its early diverging position, unique morphology for the genus and its similarities with the hypothetical ancestor of *Caenorhabditis*.

**Keywords:** Taxonomy, Systematics, Evolution, Genome, Phylogeny, Description

## Background

The nematode genus *Caenorhabditis* includes the well-known model organism *C. elegans*, which has provided key insights into molecular and developmental biology [1]. Over the past ten years, numerous new *Caenorhabditis* species have been discovered and described [2, 3]. These putative new taxa are generally indistinguishable morphologically, and thus the most recent descriptions of new species within *Caenorhabditis* have been based on DNA sequences and mating tests only [2]. This streamlined species-description methodology has been driven by the need to have names to attach to real biological entities, and the fact that traditional taxonomy

has been unable to keep up with species discovery. The method is relatively simple to implement, and delivers taxa that have a biological reality [2]. However, as the number of species discovered in *Caenorhabditis* grows, traditional, morphological descriptions are still valuable for the understanding of patterns of trait evolution and inference of ecological functions [4, 5]. Although morphology cannot be used to definitively delineate species, it should not be abandoned all together.

M-A Félix, C Braendle and AD Cutter [2] provided new species name designations for 15 biological species, considerably increasing the number of named *Caenorhabditis* species in laboratory culture. However, several key *Caenorhabditis* species remain undescribed. A well-known but undescribed species of *Caenorhabditis*, informally referred to as *Caenorhabditis* sp. 1, has been analysed in several evolutionary and developmental studies [3, 6–8]. *C. sp. 1* was previously found only once inside a fruiting body of the fungus *Ganoderma applanatum* (Pers.) Pat.

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(Polyporaceae), growing on the stump of tree in Berlin, Germany. Galleries inside the fungus were frequently visited by beetles of the species *Cis castaneus* (Ciidae), a beetle with a host preference for *Ganoderma* [9]. Associations between nematodes and insects, where the nematode uses the insect as a transport carrier (phoretism), have already been described for several *Caenorhabditis* species, including *Caenorhabditis angaria*, *C. remanei*, and *C. bovis*, and similar phoretic associations could be expected for many or possibly all other *Caenorhabditis* species [10].

Here we use both morphological and molecular analyses to characterise and describe *C. sp. 1* as a new species, *Caenorhabditis monodelphis* sp. n., and explore its relationship with the beetle *Cis castaneus*. Molecular phylogenetic analysis based on whole genome sequencing of an inbred derivative of the type strain affirms the placement of *C. monodelphis* sp. n. as sister to other analysed *Caenorhabditis*, and we analyse the evolution of phenotypic traits to infer those present in the hypothetical ancestor of *Caenorhabditis*.

## Methods

### Isolation and culture

*Caenorhabditis monodelphis* sp. n. (strain SB341) was originally isolated from fruiting bodies of *Ganoderma applanatum* (Pers.) Pat. 1887 collected in Berlin-Grunewald, Germany (April, 2001) and later from four locations in Belgium (strain DSC001 collected from 51° 06'24"N, 3°18'13"E, March 2014, strain DSC002 collected from 50°52'7"N, 4°06'54", February 2014, and an uncultured population 51°02'41"N, 3°27'17" June 2014) and from one location in the Botanical Garden in Oslo, Norway (strain JU2884; 59°55'04"N 10°46'01"E, 22 July 2015). These collections were from the same mushroom species. We also found *C. monodelphis* sp. n. in the fruiting body of *Fomes fomentarius* (L.) Fr. 1849 (50°43'02"N, 4°05'06"E, February 2015). Strain SB341 was chosen as type.

Nematodes were extracted from the fruiting bodies of *G. applanatum* using the modified Baermann method [11]. Dauer larvae were isolated from the beetle *Cis castaneus* (Herbst, 1793) that had been extracted from the same mushroom from multiple locations (except the type population and from 51°02'41"N, 3°27'17"). Adults and dauer larvae were picked out and cultured on nutrient agar plates seeded with *E. coli* OP50 at 15 °C.

### Morphological characterisation

Cultures of nematodes from two populations (strain SB341 and DSC001) were used for the description. Measurements and drawings were made with an Olympus BX51 equipped with differential interference contrast (DIC). Light microscopic images were taken with a Nikon DS-Fi2 camera. For Scanning Electron Microscopy (SEM), two fixation methods were used. For the first fixation method, live animals were fixed in a microwave in Trump's

fixative (2% paraformaldehyde + 2.5% glutaraldehyde in a 0.1 M Sorenson buffer) for a few seconds. Specimens were subsequently washed three times in double-distilled water. For the second method, specimens were put in a refrigerator at 4 °C for 1 h, then Trump's fixative was added and specimens were left overnight at 4 °C. The specimens were then washed with a 0.2 M phosphate buffer followed by 1 h post-fixation in a 1% OsO<sub>4</sub> solution at room temperature and subsequently washed 4 times in double-distilled water. For both methods, the specimens were dehydrated by passing them through a graded ethanol concentration series of 30, 50, 75, 95% (20 min each) and 3x 100% (10 min each). The specimens were critical point-dried with liquid CO<sub>2</sub>, mounted on stubs with carbon discs and coated with gold (25 nm) before observation with a JSM-840 EM (JEOL, Tokyo, Japan) at 15 kV. Sperm cells were observed in the female post-uterine sac with Transmission Electron Microscopy (TEM), processing samples as described [12], except for ultramicrotomy with a Leica EM UC7 and 1 h 1% osmium postfixation (Slos et al. unpublished).

### Molecular characterisation

For DNA barcoding analyses, temporary slides of individual nematodes were made in tap water and digital light microscope pictures were taken as a morphological voucher. The nematode was then transferred to a PCR tube with a solution containing 10 µl 0.05 M NaOH and 1 µl Tween20, heated for 15 min at 95 °C, and 40 µl of double-distilled water was added. PCR was carried out targeting either the 28S (large subunit) ribosomal RNA gene (nLSU) or the ribosomal internal transcribed spacer 2 (ITS2) locus, and PCR products were cleaned and sequenced directly. Forward and reverse primers for the nLSU were D2Ab (ACAAGTACCGTGAGGGAAAGTTG) and D3b (TCGGAAGGAACCAGCTACTA). For ITS2 we used VRAIN2F (CTTTGTACACACCGCCCGTCGCT) and VRAIN2R (TTTCACTCGCCGTTACTAAGG GAATC). The sequences obtained were 100% identical to published sequences for *Caenorhabditis* sp. 1 [3].

### Genome sequencing

Genomic DNA was extracted from an inbred strain, JU1667, of *C. monodelphis* sp. n. (derived from strain SB341), maintained on *E. coli* OP50, using the proteinase K-spin column protocol (detailed in Additional file 1). Total RNA from the same culture was also extracted (methods detailed in Additional file 1). Two paired-end genomic libraries (insert sizes of 300 bp and 600 bp, respectively) and a single paired-end RNA-seq library (insert size 180 bp) were constructed using TruSeq reagents and sequenced on the Illumina HiSeq 2000 by Edinburgh Genomics. We obtained 124.3 million genomic read pairs (100 base, paired end) and 46.2 million pairs of RNA-Seq reads (also 100 base, paired end).

### De novo genome assembly and gene prediction

Details of software versions and parameters are available (see Additional file 2). We performed initial quality control of our genomic sequence data using FastQC [13] and used Skewer [14] to remove low quality (Phred score < 30) and adapter sequence. Using blobtools [15], we generated taxon-annotated GC-coverage (TAGC) plots to identify and remove bacterial contamination. Sequence data were assembled with CLC assembler (CLCBio, Copenhagen, Denmark) and reads mapped back to this assembly using CLC mapper. Each assembly contig was compared to the NCBI Nucleotide (nt) database using megablast from the NCBI BLAST+ suite [16]. Genomic read pairs were aligned to genome references from five *E. coli* (strains: BL21 (DE3), ETEC H10407, K12 substr. DH10B, K-12 substr. MC4100 and B str. REL606) using Bowtie [17], and aligned pairs discarded. We identified laboratory-induced contamination with *Caenorhabditis elegans* in the 600 bp insert library data. To remove this, we aligned read pairs of the uncontaminated 300 bp-insert library to the *C. elegans* N2 reference genome. Regions of similarity between the genomes of *C. monodelphis* sp. n. and *C. elegans* (i.e. those regions of *C. elegans* with aligned *C. monodelphis* sp. n. reads) were masked with Ns using BEDtools [18]. Read pairs of the 600 bp-insert library were subsequently aligned to this masked *C. elegans* reference and any aligned read pairs discarded.

Cleaned sequence data were assembled with ABySS [19] ( $k = 83$ ) and contigs were scaffolded with transcript evidence using SCUBAT [20]. RepeatModeler [21] was used to identify repetitive regions which were then masked using RepeatMasker [22]. RNA-Seq read pairs were aligned to the assembly using STAR [23], and the resulting BAM file was used to guide the prediction of protein-coding genes by BRAKER [24].

### Gene structure comparisons

Genome sequences and annotation GFFs were downloaded from WormBase [25] and imported into a custom Ensembl database (version 84) [26]. Using the Ensembl Perl API, the canonical transcript from each protein-coding gene was identified and exon and intron statistics were calculated. To compare the gene structures of *C. monodelphis* sp. n. with that of *C. elegans*, we identified all orthologous clusters (details below) in which *C. monodelphis* sp. n. and *C. elegans* proteins were present as single-copy. Exon and intron statistics were calculated for each gene pair, as described previously. Plots were generated using the ggplot2 package [27] and GenePainter [28].

### Phylogenetic analyses

Pairwise comparisons of protein sequences derived from genomic data for 23 species of *Caenorhabditis* and two

outgroup species, *Oscheius tipulae* and *Heterorhabditis bacteriophora*, (see Additional file 3 for details) were performed using NCBI BLAST+ [16] and clustered into orthologous groups using OrthoFinder [29]. The sequences of 303 one-to-one orthologues (allowing for up to two species to have missing data) were extracted and aligned using ClustalOmega [30]. Poorly aligned regions were removed from the alignments using trimAL [31] and trimmed alignments concatenated using FASconCAT [32] to yield a supermatrix. We performed maximum-likelihood (ML) analysis using RAXML [33] (PROTGTR +  $\Gamma$  substitution model) with 1,000 bootstrap replicates. Bayesian analysis was performed using PhyloBayes [34] (CAT-GTR), with two independent Markov chains, and convergence was assessed using Tracer [35].

### Nomenclatural acts

This published work and the nomenclatural acts it contains have been registered in Zoobank: <http://zoobank.org/urn:lsid:zoobank.org:pub:0E6F137B-9975-4A8E-91F2-D588A572076E>. The LSID for this publication is: [urn:lsid:zoobank.org:pub:0E6F137B-9975-4A8E-91F2-D588A572076E](http://zoobank.org/urn:lsid:zoobank.org:pub:0E6F137B-9975-4A8E-91F2-D588A572076E).

### Results

Here we provide a formal description of SB341 as the type strain of *C. monodelphis* sp. n.

*Caenorhabditis monodelphis*<sup>1</sup> sp. n. Slos & Sudhaus

= *Caenorhabditis* sp. SB341 [7]

= *Caenorhabditis* sp. SB341 and *Caenorhabditis* sp. n. SB341 [36]

= *Caenorhabditis* sp. n. 1 (SB341) and (lapse)

*Caenorhabditis* sp. n. 4 (SB341) [10]

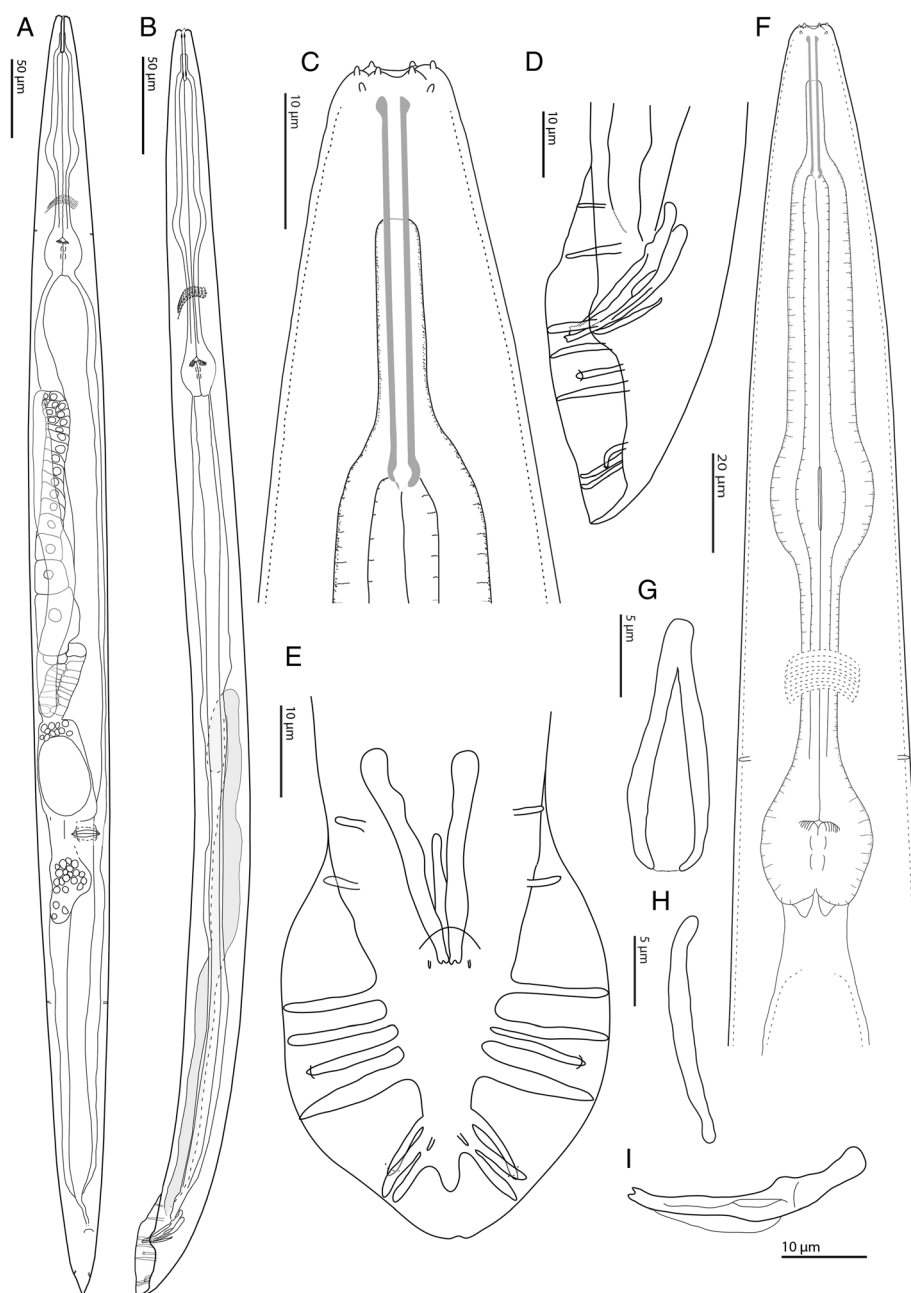
= *Caenorhabditis* sp. 1 SB341 [6, 8, 37]

= *Caenorhabditis* sp. 4 SB341 [38]

(Figs. 1, 2, 3 and 4; Table 1)

### Adult

Small species (female 0.72 - 1.04 mm, male 0.65 - 0.77 mm); cuticle thin, ca. 1  $\mu$ m wide and finely annulated, 0.8  $\mu$ m wide at midbody. Lateral field inconspicuous, about 9% of body width, consisting one ridge that can be traced anteriorly to the level of the median bulb and posteriorly at level of rectum in females and about 1½ spicules length anterior of the cloacal aperture in males. Six lips slightly protruding, each with one apical papilliform labial sensillum and a second circle of four sublateral cephalic sensilla in both sexes; amphids opening on the lateral lips, hardly discernible. Buccal tube long and slender, more than twice the width in lip region, pharyngeal sleeve envelopes nearly half of the stoma, the anterior as well as the posterior end of the tube appear slightly thickened, cheilostom inconspicuous, arcade cells forming the

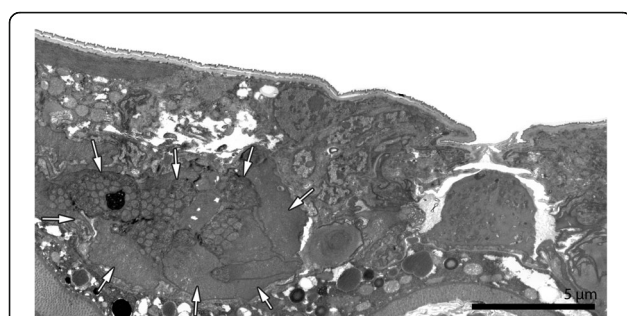


**Fig. 1** Line drawings of *Caenorhabditis monodelphis* sp. n. **a** Female, schematic overview. **b-i** Male; **b**: Male, lateral; **c** Anterior end in ventral view; **d** Tail in lateral view; **e** Tail in ventral view; **f** Pharyngeal region; **g** Gubernaculum in ventral view; **h** Gubernaculum in lateral view; **i** Spiculum in lateral view

gymnostom sometimes visible; glottoid apparatus completely absent. Pharynx with a prominent median bulb, diameter more than 90% of diameter of terminal bulb; terminal bulb pyriform, with double chambered haustulum, the anterior chamber smallish; cardia conspicuous, opens funnel-like in intestine. Nerve ring encircles isthmus in its anterior part in living specimens, more to the middle of the isthmus in heat relaxed or preserved specimens; deirids usually conspicuous in the lateral field at level

of beginning of terminal bulb, sometimes not visible in heat relaxed animals; pore of excretory-secretory system hard to discern posterior of deirid level. Two gland cells ventral and slightly posterior of terminal bulb conspicuous in live specimens. Lateral canals visible in live specimens extending anteriorly to two stoma length from the anterior end and ending at rectum level in the female. Postdeirids usually very conspicuous dorsally of the lateral field at about 75% of body length in both





**Fig. 2** *Caenorhabditis monodelphis* sp. n., mature spermatozoa in female post-uterine sac, TEM. Arrows = sperm cells

sexes and about half the length between vulva and beginning of rectum (or at level of posterior end of uterus remnant) in females, sometimes not visible in heat relaxed specimens.

### Female

Maximum body diameter clearly anterior of the vulva, vulva position 65% body length, a transverse slit, bordered in both ends by cuticular longitudinal flaps, vulva lips moderately protruding, four diagonal vulval muscles conspicuous; one pseudocoelomocyte exists anterior of gonad flexure ventrally. Genital tracts asymmetrical; posterior branch rudimentary, sac like, on the left hand side of intestine, without flexure, almost as long as body diameter at the level of the vulva, containing spermatozoa (Fig. 2); anterior branch right of intestine, reflexed dorsally close to the pharynx, flexure more than half the length of the gonad (measured from vulva to flexure); at the flexure oocytes in several rows, downstream in one row, oocytes predominantly growing in the last position, where granules are stored inside; sphincter between oviduct and uterus, only a few sperm cells in oviduct, most of them in uterus and blind sac; oviparous, one egg at a time in uterus (rarely two), segmentation starts in the uterus. Rectum a little S-shaped, rectal gland cells very small, posterior anal lip slightly protuberant. Tail short, panagrolaimid, dorsally convex, with offset tip tapering, smooth to somewhat telescope-like by cuticle forming a sleeve-like structure; tail tip with tiny hooks, mostly one dorsal, but also subventral (compare with *Poikilolaimus*); opening of phasmids located at 60–65% of tail length, shortly anterior of tip, phasmid glands not reaching anus level.

### Male

Testis right of intestine, ventrally reflexed in a certain distance posterior of pharynx; flexure relatively short. One pseudocoelomocyte between pharynx and flexure ventrally. Bursa well developed, peloderan, anteriorly open, with smooth margin and sometimes terminally indented, posterior part of velum transversely striated.

Nine pairs of genital papillae (GP) present, two of them anterior of the cloaca, genital papilla 1 (GP1) and GP2 spaced, GP3 to GP6 and GP7 to GP9 clustered, GP5 and GP7 point to the dorsal side of the velum, GP6 slightly bottle shaped, GP8 and GP9 fused at base, GP2 and GP8 not reaching the margin of velum. Phasmids forming small tubercles to the ventral side posterior of the last GP; formula of GPs: v1,v2/(v3,v4,ad,v5) (pd,v6,v7)ph. Precloacal sensillum small, precloacal lip simple (according to type A of W Sudhaus and K Kiontke [39]), post-cloacal sensilla long filamentous. Spicules short and stout, tawny, separate, slightly curved, with prominent head; shaft with a transverse seam, with a prominent longitudinal ridge, a dorsal lamella, and an oval “window”, the tip notched. Gubernaculum dorsally projecting, flexible, in the distal part following the contour of the spicules, spoon shaped in ventral view.

### Dauer larva

Unsheathed, mouth closed; stoma long, slender. Pharyngeal sleeve covering about half of the stoma; pharynx with well-developed median and terminal bulbs; corpus length ca. 52% of pharynx length. Nerve ring somewhat in the middle between the middle and terminal bulb. Genital primordium at about 60% of body length, elongated oval in shape. Tail conical. Amphids, lateral lines, position excretory pore, deirids and phasmids not observed.

### Aberration

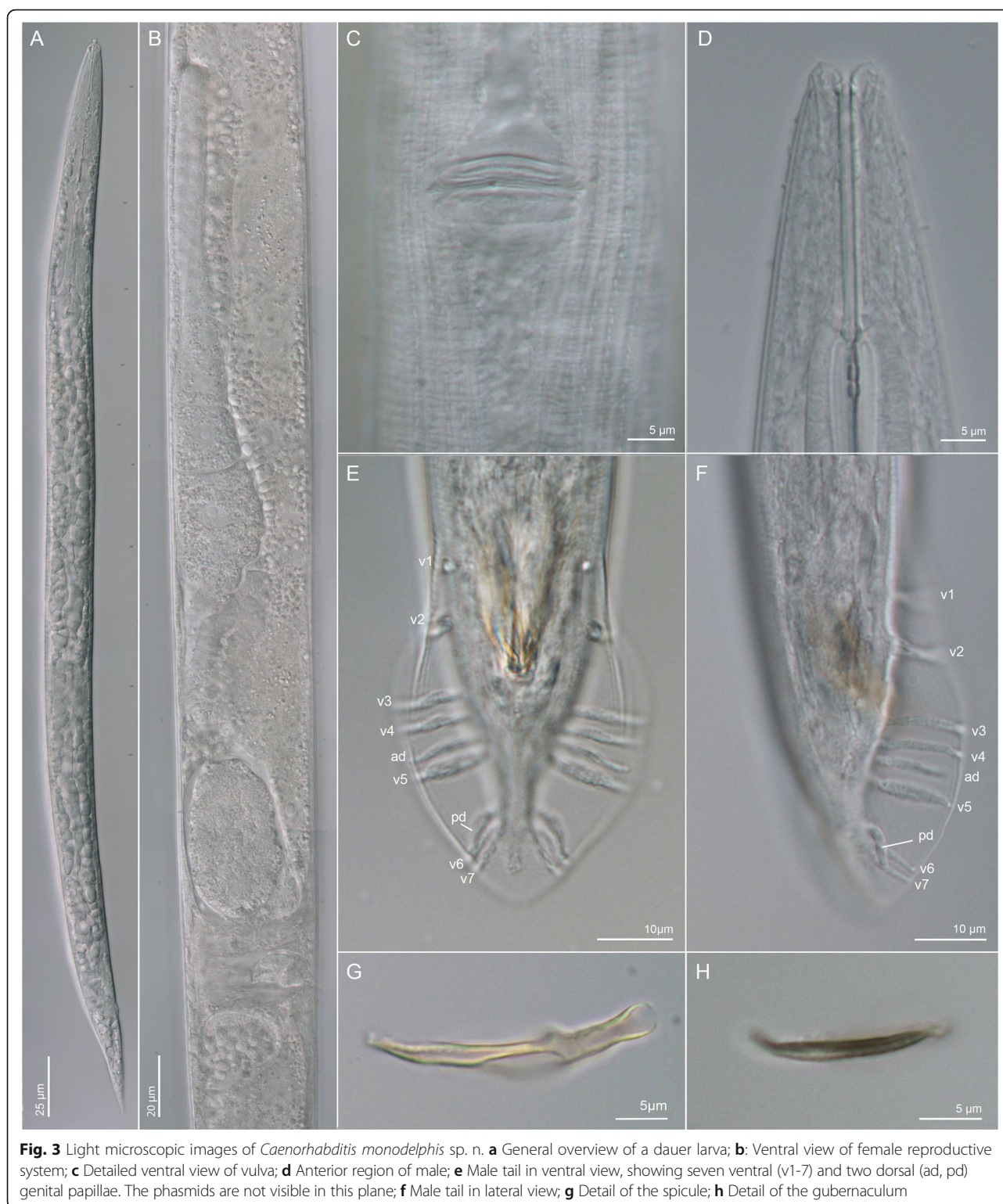
In one female a second set of “sensilla” were observed a short distance posterior to postdeirids, possibly a duplication of the postdeirids.

### Type carrier and locality

Holotype and paratypes of *Caenorhabditis monodelphis* sp. n. were isolated from the tunnels of *Cis castaneus* (Herbst, 1793) (Ciidae, Coleoptera) in the bracket fungus *Ganoderma applanatum* (Polyporales) on a stump of the common beech (*Fagus sylvatica*) a few centimetres above the ground in Berlin-Grunewald in April 2001. The same sample included individuals of *Diploscapter* sp., *Plectus* sp., *Oscheius dolichura* and one individual dorylaimid and mononchid.

### Type material

Holotype male (collection number WT 3684) and five female and four male paratypes (WT 3685, WT 3686) are deposited in the National Plant Protection Organization Wageningen, The Netherlands. In addition, four female and four male paratypes, are deposited in the collection of Museum Voor Dierkunde at Ghent University, Ghent, Belgium, five female and three male paratypes in Museum

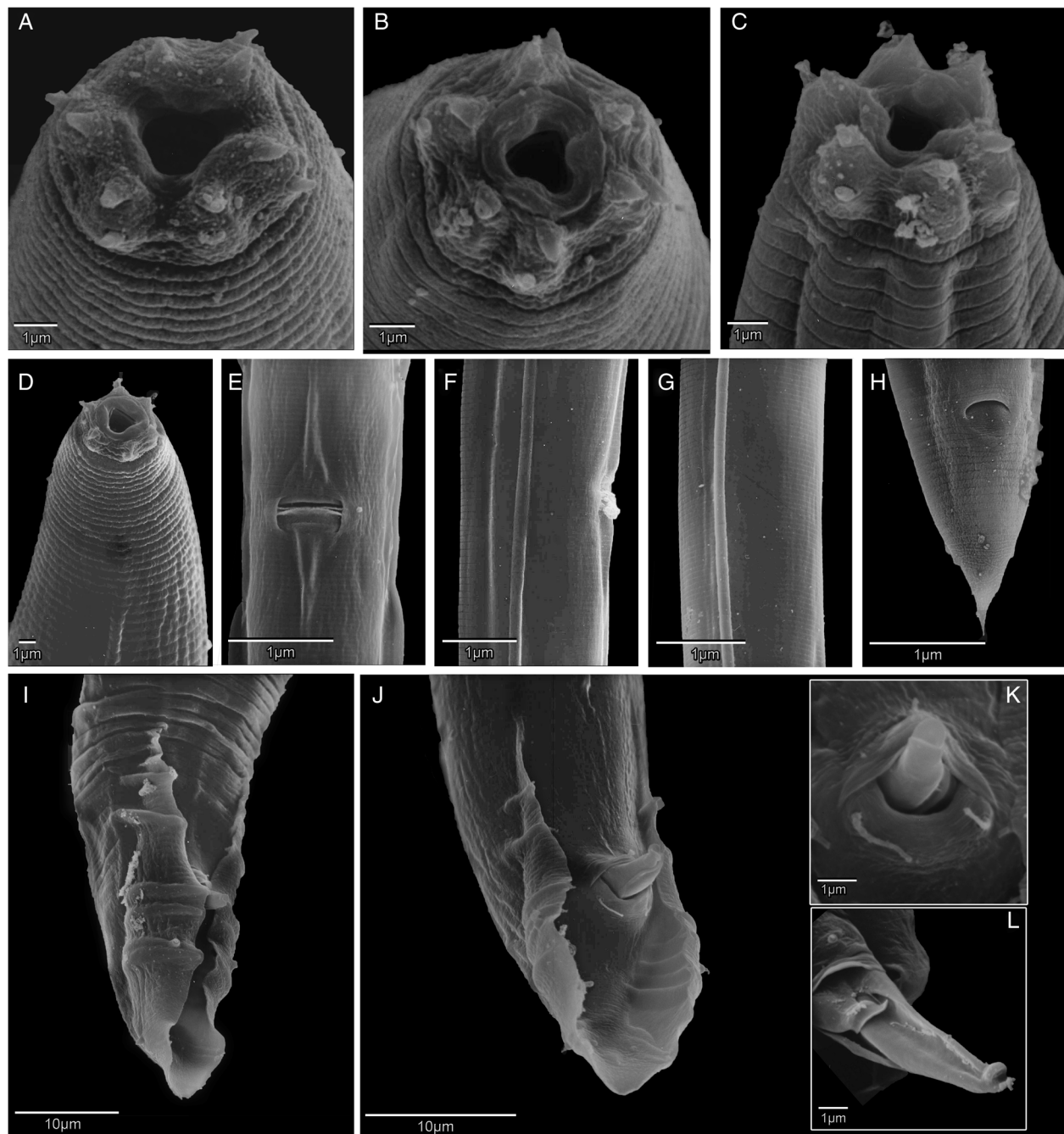


für Naturkunde an der Humboldt-Universität zu Berlin, Berlin, Germany. Additional paratypes are available in the UGent Nematode Collection (slides UGnem158, 159 & 160) of the Nematology Research Unit, Department of Biology, Ghent University, Ghent, Belgium.

#### Diagnosis and relationship

*Caenorhabditis monodelphis* sp. n. can be recognised as a *Caenorhabditis* based on the thickened GP6 and the clearly visible postdeirids. *Caenorhabditis monodelphis* sp. n. is distinguished from all other described *Caenorhabditis*





**Fig. 4** Scanning electron micrographs of *Caenorhabditis monodelphis* sp. n. **a,d** Anterior end of female; **b,c** Anterior end of male; **e** Female vulva; **f,g** Lateral field of female and male, respectively; **h** Female anus and tail; **i,j** Male bursa with genital papillae indicated; **k** Detail of cloacal region with postcloacal sensillae; **l** Detail of male spiculum

species by the presence of a monodelphic genital tract in the female with a blind sac posterior the vulva, a panagrolaimid female tail shape, adults with only one ridge on the lateral field, a very long and slender stoma without visible glottoid apparatus and male with short, stout spicule with bifurcate tip.

#### Ecology and biology

*Caenorhabditis monodelphis* sp. n. is a gonochoristic species with both males and females. Females are oviparous and carry only one egg (rarely two eggs). Development from egg to adult took about 5–6 days in juice prepared from brown algae at room temperature. Development



**Table 1** Measurements (in  $\mu\text{m}$ ) of heat relaxed specimens of *Caenorhabditis monodelphis* sp. n.

Character	Female	Male	Dauer
N	11	10	10
L	870 $\pm$ 105	694 $\pm$ 36	456 $\pm$ 24
A	17.1 $\pm$ 0.8	22 $\pm$ 1.6	23 $\pm$ 1.2
B	4.9 $\pm$ 0.5	4.1 $\pm$ 0.3	3.6 $\pm$ 0.1
C	20.5 $\pm$ 2.6	22 $\pm$ 2.3	9.8 $\pm$ 0.7
c'	1.99 $\pm$ 0.17	1.8 $\pm$ 0.2	3.9 $\pm$ 0.30
V	65 $\pm$ 1.8	-	-
Body width	51 $\pm$ 6.9	32 $\pm$ 3	20 $\pm$ 0.6
Stoma length	27 $\pm$ 2.3	27 $\pm$ 2	21 $\pm$ 1.1
Stoma diameter	1.9 $\pm$ 0.6	1.2 $\pm$ 0.2	0.6 $\pm$ 0.1
Cheilostom	2.5 $\pm$ 0.2	2.4 $\pm$ 0.2	-
Gymnostom	10 $\pm$ 0.7	9.7 $\pm$ 0.9	-
Stegostom	15 $\pm$ 1.5	15 $\pm$ 1.5	-
Pharyngeal sleeve	12.42 $\pm$ 1.6	13 $\pm$ 1.2	-
Pharynx length	150 $\pm$ 6.7	141 $\pm$ 9.2	107 $\pm$ 3.3
Procorpus length	55 $\pm$ 3.1	52 $\pm$ 3.6	-
Metacorpus length	26 $\pm$ 2.1	22.8 $\pm$ 1.1	-
Isthmus length	39 $\pm$ 3.2	40 $\pm$ 4.9	-
Nerve ring to terminal bulb	11 $\pm$ 4.9	19 $\pm$ 3.3	-
Terminal bulb length	30 $\pm$ 1.8	27 $\pm$ 1.7	-
Diameter of median bulb	22 $\pm$ 2.5	17 $\pm$ 1.3	9 $\pm$ 0.5
Diameter of terminal bulb	25 $\pm$ 2	19 $\pm$ 1	11 $\pm$ 0.4
Anterior end to deirid	150 $\pm$ 8	150 $\pm$ 8.3	-
Postdeirid to anus	170 $\pm$ 29.8	141 $\pm$ 14	-
Length intestine	651 $\pm$ 100	494 $\pm$ 32	-
Rectum length	25 $\pm$ 2.6	24 $\pm$ 1.9	-
Anal body width	22 $\pm$ 2.1	17 $\pm$ 1.1	12 $\pm$ 0.6
Tail length	43 $\pm$ 4.3	32 $\pm$ 3.2	46 $\pm$ 2.4
Anus to phasmid distance	26 $\pm$ 2.2	-	-
Gonad length <sup>a</sup>	303 $\pm$ 68	342 $\pm$ 44	-
Gonad flexure length	226 $\pm$ 67	46 $\pm$ 6.8	-
Postuterine sac	45 $\pm$ 6.8	-	-
Sperm diameter	-	9.8 $\pm$ 1.3	-
Egg length <sup>b</sup>	53 $\pm$ 3.1	-	-
Egg diameter <sup>b</sup>	29 $\pm$ 2.9	-	-
Spicule length	-	25 $\pm$ 1	-
Gubernaculum length	-	15 $\pm$ 0.9	-

<sup>a</sup>from anus to flexure in the female; from cloaca to flexure in the male<sup>b</sup>n = 7

from dauer larva to adults was completed in less than 3 days at 20 °C on NA seeded with OP50. The lifespan of adults is at minimum 14 days for males and 17 days for females. One pair of adults produced 167 offspring in 8 days and the daily production of fertile eggs was 6–31

(mean 18; n = 14). After the reproductive phase, females lived 9–14 days (n = 3) with males present.

*Caenorhabditis monodelphis* sp. n. has until now only been found in *Ganoderma* and *Fomes* in Germany and Belgium in relation with the ciid beetle *Cis castaneus*. The *Ganoderma* carrying *C. monodelphis* sp. n. from Oslo was not investigated for the presence of *C. castaneus*. In fungal fruiting bodies lacking the beetle *C. monodelphis* sp. n. was not found. Dauers of *C. monodelphis* sp. n. were found under the elytra of the beetle, but were not found internally when the beetle was further dissected. These findings indicate a phoretic association with the beetle. As only dauer larvae were isolated from beetles, while adults and larvae were present in the fruiting bodies, we infer that *C. monodelphis* sp. n. exit from dauer within the mushroom, develop to adulthood and start to reproduce. The food source of the species in natural conditions is not known, but they survive and reproduce easily on *E. coli* OP50 in culture.

#### Genome sequence of an inbred strain of *Caenorhabditis monodelphis* sp. n.

We sequenced the genome of an inbred strain (JU1677) of *C. monodelphis* sp. n. using Illumina sequencing technology to ~110x coverage. The genome was assembled into 6,864 scaffolds, spanning 115.1 Mb with a scaffold N50 of 49.4 kb (Table 2). CEGMA (Core Eukaryotic Gene Mapping Approach) [40] scores suggested the assembly is of high completeness. We predicted 17,180 protein coding gene models using RNA-Seq evidence. These statistics, and the overall gene content and structure of the assembly were largely in keeping with those determined for other *Caenorhabditis* species. The genome was larger than that of *C. elegans* and *C. briggsae*, which are hermaphroditic species, but smaller than that of *C. remanei*, a gonochoristic species.

We carried out preliminary comparisons of the structure and content of the *C. monodelphis* sp. n. genome with those of other sequenced *Caenorhabditis* species. The number of genes identified was lower than estimates for most other *Caenorhabditis* species. To compare the gene structures of *C. monodelphis* sp. n. to that of *C. elegans*, we identified 6,174 orthologous gene pairs and calculated gene structure statistics (Table 3, Fig. 5.). To minimize bias from erroneous gene predictions (such as merged or split genes), orthologous gene pairs which differed in CDS length by 20% were considered outliers. *C. monodelphis* sp. n. genes were typically longer than their orthologues in *C. elegans*. We also found a clear trend toward more coding exons per gene in *C. monodelphis* sp. n. than in *C. elegans* (Fig. 5a). A few examples of *C. monodelphis* sp. n. gene models compared to those of orthologues in *C. elegans* are shown (Fig. 5b). Although introns are, on average, shorter in *C. monodelphis* sp. n. than in *C. elegans*,

**Table 2** Genome assembly statistics for *C. monodelphis* sp. n. and other *Caenorhabditis* species

Species	<i>C. monodelphis</i>	<i>C. brenneri</i>	<i>C. briggsae</i>	<i>C. elegans</i>	<i>C. japonica</i>	<i>C. remanei</i>	<i>C. sinica</i>	<i>C. tropicalis</i>
Version	1.0	WS254	WS254	WS254	WS254	WS254	WS254	WS254
Mating type	gonochoristic	gonochoristic	hermaphroditic	hermaphroditic	gonochoristic	gonochoristic	gonochoristic	hermaphroditic
Strain	JU1667	PB2801	AF16	N2	DF5081	PB4641	JU800	JU1373
Span (Mb)	115.12	190.37	108.38	100.29	166.25	118.55	130.76	79.32
Scaffolds (n) <sup>a</sup>	6,864	3,305	367	7	18,808	1,591	11,966	660
N50 (kb)	49.4	381.96	17,485.44	17,493.82	94.15	1,522.09	25,564	20,921.87
Genes (n)	17,180	30,660	21,814	20,362	29,964	26,226	34,696	22,326
GC (%)	43.9	38.6	37.4	35.4	39.2	37.9	39.5	37.7
CEGMA complete/ partial (%)	89.11/ 97.98	98.39/ 99.60	97.98/ 99.19	96.77/ 99.19	78.63/ 97.18	94.35/ 98.79	95.56/ 99.60	97.18/ 98.79

<sup>a</sup>Scaffolds shorter than 500 bp were not considered

*C. monodelphis* genes typically have a longer total span of introns than *C. elegans* transcripts (Table 3, Fig. 5.).

#### *C. monodelphis* sp. n. is sister to other known *Caenorhabditis*

We clustered a total of 634,564 protein sequences from *C. monodelphis* sp. n., twenty-two other *Caenorhabditis* species, and two rhabditomorph outgroup species (*Oscheius tipulae*; data courtesy of M. A. Félix, and *Heterorhabditis bacteriophora*) to define putative orthologues. We identified 34,425 putatively orthologous groups containing at least two members, 303 of which were either single copy or absent across all 25 species. These single copy orthologues were aligned, and the alignments concatenated and used to perform maximum-likelihood and Bayesian inference analysis using RAxML and PhyloBayes, respectively. Both analysis methods resulted in an identical topology, with the placement of *C. monodelphis* sp. n. arising basally to all other *Caenorhabditis* species (Fig. 6). All branches had maximal support except for three nodes within the *Elegans* super-group. Our analysis included data from several new and currently undescribed putative species of *Caenorhabditis*, including *C. sp. 21* which is the sister taxon to the *Drosophilae* plus *Elegans* super-groups and *C. sp. 31* which forms the first branch in the *Elegans* super-group. *C. sp. 38* is placed within the *Drosophilae* super-group, while *C. sp.*

26, *C. sp. 32* (sister to *C. afra*) and *C. sp. 40* (sister to *C. sinica*) are all members of the *Elegans* super-group. From these analyses we conclude that *C. monodelphis* sp. n. is sister to all other known *Caenorhabditis*.

#### Stemspecies pattern reconstruction

Our phylogenetic analyses were based on species with whole genome data available, and thus did not include the full known diversity of the genus. The stemspecies pattern was reconstructed based on ingroup and outgroup comparison. Previous molecular phylogenetic analyses of *Caenorhabditis* species using a small number of marker genes [10] placed *C. monodelphis* sp. n. and *C. sonoreae* [41] as sister species, again arising at the base of the genus.

The following morphological synapomorphies can be hypothesised to support a *C. monodelphis* sp. n. – *C. sonoreae* clade: mouth opening triangular (Fig. 4b), spicule having a complicated tip (notched or dentated) and a longish thin walled “window” in the blade (Figs. 1i, 4l), postcloacal sensilla being filiform (Fig. 4k), and the female tail shortened to less than three times anal body width. Other similarities between both these species are plesiomorphic.

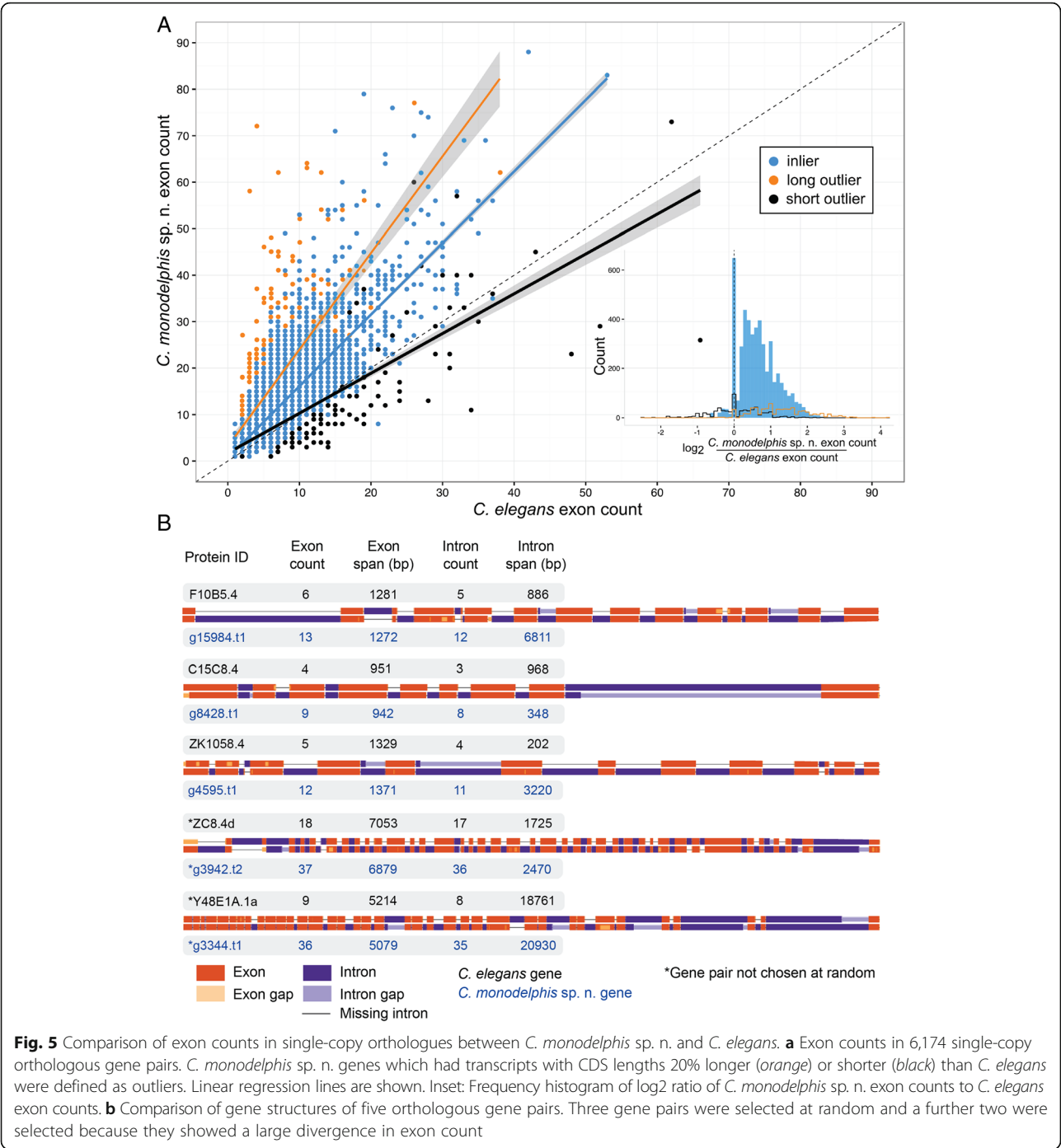
*Caenorhabditis* and its sister group constitute the monophylum Anarhabditis within the Rhabditina. For convenience, we will call the sister clade of *Caenorhabditis* Protoscapter (Fig. 7): it comprises “*Protorhabditis*”, *Prodontorhabditis*, *Diploscapter* and *Sclerorhabditis* [42]. To reconstruct the characters of the stemspecies of *Caenorhabditis* it is necessary to consider the morphologies of all these taxa, and not only the taxa for which we have molecular data. “*Protorhabditis*” is paraphyletic. The *Oxyuroides* group is sister taxon of *Prodontorhabditis* [43, 44], and the *Xylocola* group may be sister taxon of *Diploscapter/Sclerorhabditis*. However, the two species *Protorhabditis elaphri* (Hirschmann in Osche, 1952) and *P. tristis* [45] appear to represent basal branches in Protoscapter (compare [43]). These last two species, despite

**Table 3** Gene structure comparison of orthologous gene pairs from *C. monodelphis* sp. N. and *C. elegans*

	<i>C. monodelphis</i> sp. n.	<i>C. elegans</i>
Gene length (bp)	3359	2854
Coding exon length (bp)	109	144
Coding exon count (n)	10	6
CDS span (bp) <sup>a</sup>	1167	1182
Intron length (bp)	69	76
Total intron span per gene (bp)	1918	1187

All values are medians

<sup>a</sup>orthologous gene pairs which differed in CDS length by 20% were not included

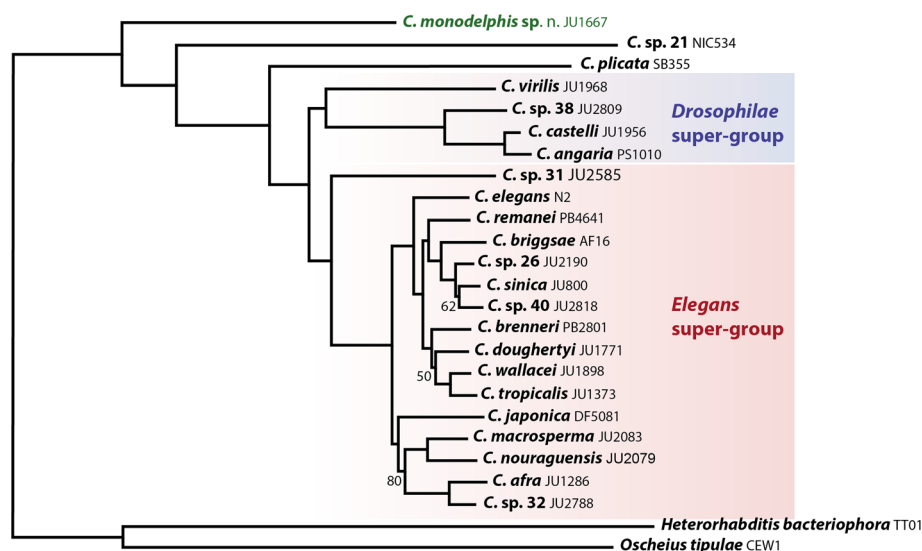


the paucity of information available for them, are crucial for comparisons that will illuminate the stemspecies patterns of *Anarhabditis*, *Protoscapter* and *Caenorhabditis*.

By ingroup comparison we reconstruct the following characters of the stemspecies of *Anarhabditis* without differentiating them into apo- or plesiomorphies (on apomorphies see the legend of Fig. 7):

- adults of small size (less than 1 mm);
- lips not offset from anterior end;
- four cephalic sensilla present in male and female;
- stoma with pharyngeal sleeve (stegostom length nearly that of gymnostom);
- median bulb of pharynx strongly developed, corpus intima with transverse ridging, terminal bulb with double haustulum;
- gonochoristic;
- female tail elongate conoid;
- gonads amphidelphic, the anterior branch right and the posterior left of intestine;
- vulva at midbody, a transverse slit;
- oviparous, usually only one egg at a time in the uteri;
- male gonad on the right side, reflexed to the ventral;





**Fig. 6** Phylogenetic relationships of *Caenorhabditis* species and two outgroup species. Maximum-likelihood based tree from RAxML. Bootstrap support values (1000 replicates) were 100 all branches, unless noted otherwise as numbers on branches. Bayesian Posterior Probabilities were 1 for all branches and are not shown. Coloured boxes indicate supergroups, as defined in [3]. Strain names from which protein sequence were derived are noted

bursa peloderan and anteriorly open, oval-shaped in ventral view, with smooth margin, terminally not notched; – 9 pairs of even genital papillae, two precloacal largely spaced, GP3–6 evenly spaced, the last three GPs forming a tight cluster; GP1, GP5 and GP7 terminate on the dorsal surface of the bursa velum; – phasmids open behind GP9, inconspicuous; – bursa formula thus v1,v2/v3,v4,ad,v5 (pd,v6,v7)ph; – male tail tip present; – 1 + 2 circumcloacal sensilla inconspicuous, precloacal lip simple; – spicules separate, stout, head not rounded, behind the shaft a slight ventral projection, dorsal part of blade weakly cuticularised (velum), its tip possibly not even (argued below); – gubernaculum simple spatulate; – dauerlarvae with double cuticle (ensheathed), not waving.

## Discussion

### Taxonomy of *Caenorhabditis monodelphis* sp. n.

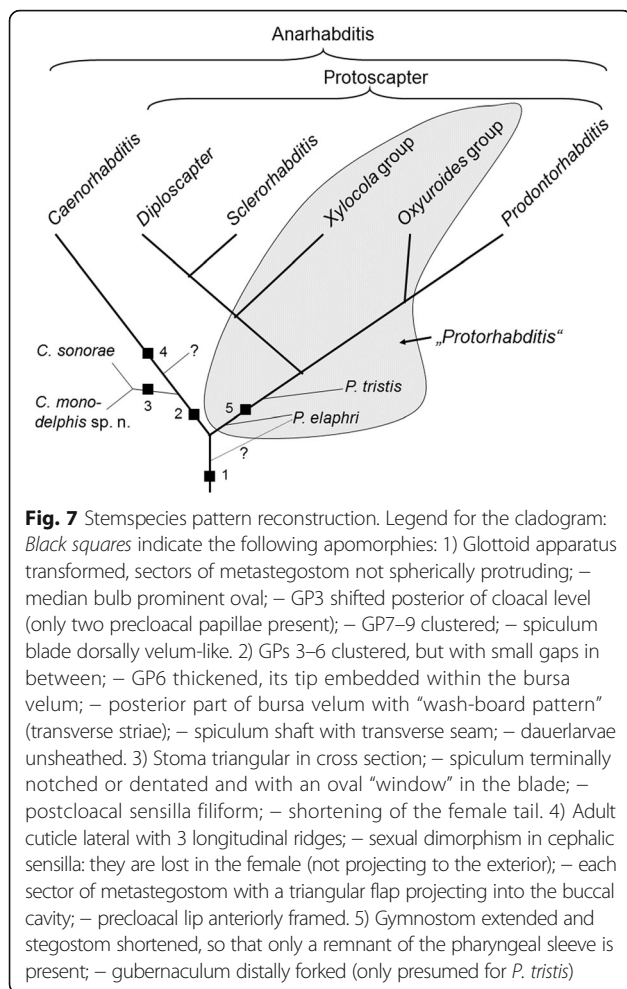
*Caenorhabditis monodelphis* sp. n. is a new species of *Caenorhabditis* supported by its phylogenetic position as inferred from 303 molecular markers, morphology, habitat and specific association with *Cis castaneus* (Coleoptera). Morphologically, it could be confused with “*Protorhabditis*” species because of the absence of a clear glottoid apparatus. A glottoid apparatus has been lost 5–6 times independently within “*Rhabditidae*” [46] and, as illustrated here, also in *C. monodelphis* sp. n. This species resembles species from “*Protorhabditis*” with a very long stoma without glottoid apparatus, but differs from the *Oxyuroides*-group within “*Protorhabditis*” in having an open bursa and GP1 not anterior of the bursa. It is differentiated from the *Xylocola*-group within “*Protorhabditis*” in having nine genital papillae.

Previously, *Caenorhabditis* has been characterised as having the following apomorphic characteristics: the presence of a dorsal velum on the spicule, a lateral field with three ridges, an unsheathed dauer juvenile and a slightly thickened GP6 [42]. With the discovery and description of *C. monodelphis* sp. n. the number of lateral ridges is no longer an apomorphic character of *Caenorhabditis*, since *C. monodelphis* sp. n. only has one lateral ridge.

### Association with fungivorous beetles

Species of *Caenorhabditis* are known to occur in soil, compost, cadavers of insects, some plant material and the intestine of birds [10], and can most easily be isolated from rotting fruits, flowers and stems [3]. *Caenorhabditis elegans* has also been found infesting cultures of the mushroom *Agaricus bisporus* [47]. Wild mushrooms are an under-explored habitat for this genus, but our limited geographical sampling indicates that they could be an important habitat. *Caenorhabditis monodelphis* sp. n. was present in galleries made by *Cis castaneus* inside *Ganoderma applanatum* in Belgium, Norway, Germany and in an old fruiting body of *Fomes fomentarius* in Belgium. Although the true distribution of *C. monodelphis* sp. n. is not yet known, it is expected that this species will be found throughout Europe where *Ganoderma* (or in lesser extent *Fomes*) co-occurs with the mycophagous beetle *Cis castaneus*.

That *Caenorhabditis* species have phoretic relationships with insects and other invertebrates is well known [10]. For *C. monodelphis* sp. n., all records are from



mushroom fruiting bodies that are also inhabited by different insect groups, and dauer larvae were found under the elytra of *Cis castaneus*. Based on this evidence, we propose that *C. monodelphis* sp. n. propagates in galleries generated by *Cisidae* and the dauer larvae are transported by these beetles to uninfested mushrooms. Records of *C. monodelphis* sp. n. in both *Ganoderma* and *Fomes*, respectively the preferred [9] and the known [48] host indicate a beetle-specific rather than a mushroom-specific relationship. The only other known *Caenorhabditis* species which appears to be phoretically associated with fungivorous organisms, most likely insects, is *C. auriculariae* Tsuda & Futai, [49] of the *Elegans* super-group. This species was found only once in the fruit bodies of *Auricularia polytricha* (Agaricomycetes) in Japan, but the vector needed to infest the mushroom is unknown [49]. *C. elegans* was also found to infest cultures of the champignon mushroom *Agaricus bisporus* [47], but most likely originated from mushroom compost where it can be frequently found. Several samples of different mushrooms on wood in Europe, USA and Japan did not yield other *Caenorhabditis* spp. However, given that many more insect species are known

to feed and reproduce on mushrooms [50] and Rhabditida are known to use insects as a phoretic transport carrier [51], it is possible that mushroom species are habitats for many other rhabditid species, including new species of *Caenorhabditis*.

#### Genome sequence and gene structures of *C. monodelphis* sp. n.

Using next generation sequencing technologies and advanced bioinformatics toolkits, we have generated a good first-draft genome sequence for an inbred line derived from the type strain of *C. monodelphis* sp. n.. Although assembly metrics and CEGMA scores indicate the assembly is relatively contiguous and complete, it is likely that a proportion of *C. monodelphis* sp. n. genes are assembled only partially. This may have affected gene prediction, with the number of predicted gene models (17,180) being lower than estimates from most other *Caenorhabditis* species with available sequence data [25]. Comparisons of orthologous gene pairs revealed a significant divergence in gene structure between *C. monodelphis* sp. n. and *C. elegans*. *C. monodelphis* sp. n. genes are typically longer, contain more coding exons and a longer span of introns than *C. elegans* genes (Table 3). This increase in gene length may, in part, account for the difference in genome span between *C. monodelphis* sp. n. and *C. elegans*. The clear trend towards more coding-exons in *C. monodelphis* sp. n. relative to *C. elegans* (Fig. 5) could be explained by extensive intron loss or gain in either species. Previous studies using a small number of genes have shown that intron losses have been far more common in *Caenorhabditis* evolution than intron gains [7, 52, 53]. Thus, it is possible that the gene structures seen in *C. monodelphis* sp. n. reflect an intron-rich ancestral state, and intron loss has predominated during the evolution of *C. elegans*. In *Pristionchus pacificus*, which is distantly related to *Caenorhabditis*, genes typically have roughly twice as many introns as their orthologues in *C. elegans* [54]. Further analysis using genomes from more closely related outgroup species and other *Caenorhabditis* species is necessary before we can infer the dynamics of intron evolution in the genus.

#### Phylogenetic analyses

Phylogenetic analysis of 303 clusters of putatively orthologous protein sequences derived from whole genome sequence data of 23 species of *Caenorhabditis* and two outgroup species resulted in a well resolved phylogenetic diagram and confirmation of *C. monodelphis* sp. n. as basal to all other analysed *Caenorhabditis* species (Fig. 6). The topology is largely congruent with previously published analyses performed using a smaller number of molecular loci [1]. However, in contrast to the analyses of Kiontke et al. [1] and Felix et al. [2] which show *C. brenneri* and *C. doughertyi* as sister species, our phylogenetic

hypothesis places *C. doughertyi* as more closely related to *C. wallacei* and *C. tropicalis*. This node, however, has low bootstrap support. Genome sequencing projects for several *Caenorhabditis* species, including those from the currently under-sampled *Drosophilae* super-group, are currently underway. These data will be essential to resolving the phylogenetic relationships of this important genus where morphology can be misinformative and/or misleading.

### Reconstruction of stemspecies pattern

Details of our inference of ASR depends on the placement of *P. elaphri* in “*Protorhabditis*” versus as sister taxon of *Anarhabditis* (because of its distinct pharynx morphology) (Fig. 7). Molecular data resolving this issue are urgently required. *Caenorhabditis monodelphis* sp. n. and *P. elaphri* share a conspicuously long and narrow stoma due to an extended stegostom (long pharyngeal sleeve) without a glottoid apparatus (bulging of the three sectors of metastegostom) and one ridge in the lateral field. Based on current evidence, we interpret these peculiar similarities as homologous and thus as further characters of the *Anarhabditis* stemspecies as well as the *Caenorhabditis* stemspecies. The narrowing of the buccal cavity could have restricted the formation of sectoral swellings of the metastegostom, so that the typical glottoid apparatus disappeared. This happened in parallel in the rhabditid *Matthesonema eremitum* [55]. The hypothesis of a reduction of the glottoid apparatus and its denticles in the stemspecies of *Anarhabditis* is in conflict with the structure of the metastegostom in most species of *Caenorhabditis*, where it looks like a transformation of a glottoid apparatus [39], and in *C. sonoreae* is credibly described as a glottoid apparatus [41]. To solve this conflict we must assume a partial reversion both in *C. sonoreae* and in the sister-lineage of *C. sonoreae/C. monodelphis* sp. n. However, instead of proposing two independent reversions, the possibility of an independent reduction of the glottoid apparatus in *Protoscapter* and *C. monodelphis* sp. n. remains an equally parsimonious alternative. A reinvestigation of *P. elaphri* could resolve this question.

In the stemspecies pattern of *Anarhabditis* the morphology of the tip of the spicules remains unclear. In the description of *P. elaphri* some drawings show the tip to be nearly pointed [45], but in other drawings (Figure twelve m of [45]) it is terminally notched. In *P. tristis*, I Andr ssy [56] depicted a small terminal hook. These characters were not mentioned in the text in either species’ description. Nevertheless, the dentation of the spicule tips in the first branching *Caenorhabditis sonoreae/C. monodelphis* sp. n. is different and distinct enough to judge this character as synapomorphic for these sister species (Fig. 7). Starting from the characters of the last common stemspecies of both these species, in *C. sonoreae* the lateral ridge must have been reduced, so that its lateral field is smooth, and

the male tail tip was retracted, so that the tail ends obtusely between the last GPs. In *C. monodelphis* sp. n. both these characters remain plesiomorphic, but the female posterior gonad branch is in the process of reduction. The ecological requirements of *C. sonoreae* (inhabitant of cactus rot) and *C. monodelphis* sp. n. (living in the tunnels of Ciidae beetles in bracket fungi) are so different, that no statement on the ecology of their last common stemspecies is possible. However, as *P. elaphri* and *C. monodelphis* sp. n. exhibit a phoretic relationship with beetles and their dauer larvae seek a place under the elytra, we cautiously suggest that this behaviour could be found in the stemspecies of *Anarhabditis* and that of *Caenorhabditis*, respectively.

Transformations from the stemspecies pattern of *Anarhabditis* to *Caenorhabditis* can be traced in the cladogram (Fig. 7). With respect to the hypothesis of the stemspecies pattern of *Caenorhabditis* formulated by W Sudhaus and K Kiontke [39] only the character of the lateral field must be revised: a single ridge in the lateral field of adults must be assumed in the stemspecies pattern of *Anarhabditis* and of *Caenorhabditis*, respectively. Therefore, the evolution of three lateral cuticular ridges must have occurred first within *Caenorhabditis* (Fig. 7).

### Degenerative evolution towards monodelphy

Uniquely for *Caenorhabditis* species, in *C. monodelphis* sp. n. the posterior female gonad branch has been reduced to a blind sac without gamete forming function. This vestigial branch serves mainly in storing sperm. In contrast to most mono-prodelphic rhabditids, the vulva is not shifted posteriorly in *C. monodelphis* sp. n.. A relict posterior gonad together with a nearly median vulva also occurs in *Oscheius guentheri* (Sudhaus & Hooper, 1994) [57] and an undescribed *Diplogastrellus* species from India (Sudhaus, unpublished data). Remarkable, in all these cases the anterior branch does not extend into the body posterior to the vulva, in contrast to monodelphic cephalobids, panagrolaimids and the rhabditid *Rhabpanus*. In *Rhabpanus ossiculus* Massey, [58] and *R. uniuus* Tahseen, Sultana, Khan & Hussain, [59] the prodelphic reflexed gonad reaches almost to the rectum while the vulva is located at 65–69% of body length and a short post-uterine sac filled with sperm is present [58, 59]. In contrast to species of *Acroboloides*, *Cephalobus*, *Mesorhabditis* and *Panagrolaimus*, the posterior branch of the gonad of *O. guentheri* is not reduced by apoptosis of the distal tip cell [60], and the vestigial branch is very variable within this species [57]. These patterns argue for a relatively recent reduction in *O. guentheri*. Based on the similarities (in the female gonad and nearly median vulva) between *C. monodelphis* sp. n. and *O. guentheri*, the gonadal system of female *C. monodelphis* sp. n. may also represent a relatively recent evolutionary shift.



## Conclusions

The basal position and the unique characters of *C. monodelphis* sp. n. in the genus *Caenorhabditis* and its similarity with the hypothetical ancestor of *Caenorhabditis* makes *C. monodelphis* sp. n. a key species for future evolutionary and developmental studies within *Caenorhabditis*. Importantly we present here, alongside traditional morphological diagnosis of this new species a complete genome draft, which we believe is the first time this has been done for a metazoan species description. Release of the draft genome sequence of *C. monodelphis* sp. n., along with its formal description will, we hope, promote forward- and reverse-genetic analyses of its biology. In particular, CRISPR-Cas9 gene editing technologies, which require sequence knowledge for design of targeting oligonucleotides, are immediately facilitated.

While publication of marker sequence alongside species description is becoming commonplace [61], formal publication of whole genome data alongside species descriptions has historically been limited to prokaryotic taxa. In Eukaryota, this practice is just gaining traction, with the recent publication of the description of a fungal taxon with genome data (*Epichloë inebrians*, an ergot fungus [62]). Additionally, novel arthropod taxa used in phylogenomic analyses have had species descriptions published independently, but near-concurrently, with their genome data (*Mengenilla moldrzyki*, a strepsipteran insect [63, 64] or transcriptome data (the centipede *Eupolybothrus cavernicolus* [65]). For *Caenorhabditis* species, where morphology can be misinformative and/or misleading, phylogenomic analyses – and thus determination of genome sequence – will be essential for resolution of relationships. We suggest that genome scale data allied to species description should become commonplace.

## Endnote

<sup>1</sup>Named after the monodelphic reproductive system in the female.

## Additional files

**Additional file 1:** Nucleic acid isolation from *Caenorhabditis monodelphis* sp. n strain JU1677. (DOCX 13 kb)

**Additional file 2:** Details of software, versions and parameters used in analysis. (TSV 5 kb)

**Additional file 3:** Accessions and links to genome-derived protein sequence data used in phylogenetic analysis. (TSV 4 kb)

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## Availability of data and materials

Genome and transcriptome sequence read data of *C. monodelphis* sp. n. JU1677 are available from the European Nucleotide Archive and NCBI Short Read Archive under the accession PRJEB7905. The genome assembly and annotations are available to browse and download at [ensembl.caenorhabditis.org](http://ensembl.caenorhabditis.org) and [download.caenorhabditis.org](http://download.caenorhabditis.org), respectively. Accessions and links to genome-derived protein sequence data used in phylogenetic analyses are available in Additional file 3. Data files associated with this study have been deposited in Zenodo under the accession 10.5281/zenodo.160693.

## Authors' contributions

DS, WS, LS and MB conceived the study. DS, WS, LS collected and analysed data and wrote the manuscript. All authors provided comments on early drafts of the manuscript. WB, LS and MB funded this study. All authors read, revised, and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

## Consent for publication

Not applicable.

## Ethics approval and consent to participate

Not applicable.

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